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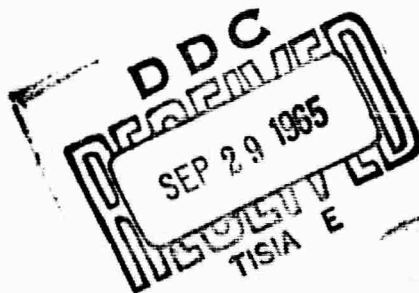
ELECTROSTATIC FORCES IN DYNAMIC FLUORESCENCE QUENCHING

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Abstract - The quenching constant for the iodide quenching of the fluorescence of riboflavin phosphate was measured at pH 5 and at pH 8. The value of the constant at the lower pH (where the phosphate group is unionized), $\underline{k}' = 23$ M^{-1}, was independent of the ionic strength, μ. The ratio $\underline{k}''/\underline{k}' \equiv \underline{f}$ ($\underline{k}''$ the quenching constant at pH 8, where the phosphate group carries a single negative charge) was found to be 0.70 at $\mu = 0.025$ and 0.79 at $\mu = 0.045$. The fact that $\underline{f} < 1$ is interpreted as due to electrostatic repulsion between the dye molecule, when ionized, and the iodide ion. Due to counter ion screening the effect is less accentuated at higher μ. The quenching constant for riboflavin (which does not contain the ionizable phosphate group) was found to have the same value, 30 M^{-1}, both at pH 5 and at pH 8, and with no dependence on μ.

Introduction

Dynamic (as opposed to static) fluorescence quenching is a process where the excitation energy of an excited dye molecule is removed on diffusional encounter with another molecule, called the quencher, Q. For comparatively low quencher concentrations, the quenching follows the Stern-Volmer equation:

$$\frac{I_o}{I} - 1 = k/Q/ \tag{1}$$

where I_0 is the fluorescence intensity (in arbitrary units) in absence of quencher, I the same at quencher concentration $/Q/$ (mole/l, M) and k is the "quenching constant" (M^{-1}). At higher values of $/Q/$, positive deviations from the Stern-Volmer line are normally observed.

Based on the concept of diffusionally controlled reactions, several theories have been advanced to predict the quenching constant from molecular parameters such as τ_0 (the fluorescence lifetime in absence of quencher), D (the sum of the diffusional constants of the dye, D_A, and of the quencher, D_Q), a (the centre-to-centre distance between excited dye and Q, over which quenching can occur), and γ (the probability that an encounter between excited dye and Q will lead to quenching). If both the dye and the quencher are ions (with charges z_A and z_Q, resp.), the quenching constant is expected to either decrease (if $z_A \cdot z_Q > 0$, i.e. repulsion) or increase (if $z_A \cdot z_Q < 0$, i.e. attraction). These effects should be less pronounced at higher ionic strengths, where the Coulomb forces are partially masked by counter-ion screening.

The ability of an ionic quencher, e.g. I^-, to quench the fluorescence of a dye will in general be different in a pH region where the dye is neutral than in a pH region where it is ionized. If, however, the ionization takes place directly on the resonant system of the dye, one really has to deal with two different dyes in the two cases and the observed change in quenching constant cannot directly be interpreted as due entirely to electrostatic forces.

Riboflavin consists of an iso-alloxazine group and a ribityl group, neither carrying any net charge in the pH region 3 to 9. In riboflavin phosphate (flavin mono nucleotide, FMN), the

phosphate group, attached to the end of the ribityl group, has a pK_a of 6.6 /2/. At pH 5, the whole molecule is neutral, but at pH 8 it carries a negative charge located at the phosphate group, well isolated from the light-absorbing and -emitting system (the iso-alloxazine group). The differences between the quenching effect of iodide on riboflavin phosphate fluorescence at pH 5 and pH 8, as reported below, should then be due only to the electrostatic repulsion in the latter case. For riboflavin (not carrying the ionizable phosphate group), no difference in the quenching constant was found comparing the two pH values.

Experimental

Riboflavin was obtained from the British Drug Houses, riboflavin-5'-phosphate (as sodium salt) from California Corporation for Biochemical Research. The purity of the two dyes was tested by paper chromatography (Munktell No. 302, corresponding to Whatman No. 1, n-butanol - acetic acid - water as eluant). No degradation products were detected, and the dyes were used without further purification. Other chemicals were of analytical grade.

Solutions were made up from triply distilled water and buffered to the desired pH with phosphates at a constant total concentration of 5 mM. The concentration of the dye was kept constant at 10 μM; at this low concentration, self-quenching and reabsorption of the fluorescence are negligible. In order to keep the ionic strength constant while varying the concentration of quencher (added as potassium iodide), sodium chloride was added.

The fluorescence intensity was measured with a Zeiss spectrofluorometer, model ZFM4C, with a xenon arc lamp (Osram

XBO 450) plus a monochromator as exciting light source, and a second monochromator plus a photomultiplier as emission detector. Maximum signal was obtained with the first monochromator at either 470 mμ or 375 mμ, and the second monochromator at 540 mμ. These settings were used for the measurements; as only intensity ratios were of interest, no corrections were applied for the wavelength variation of the xenon lamp emission or for the sensitivity of the photomultiplier. The measurements were performed at room temperature (22° C).

Results

The fluorescence intensity of riboflavin phosphate, in absence of quencher (I_o) was practically the same at pH 5 and at pH 8, and at all ionic strengths studied, and also practically equal to the fluorescence intensity of riboflavin under the same conditions. The ratios I_o/I were independent of the wavelength, λ_1, used for excitation, but I_0 was about 4 times higher for $\lambda_1 = 470$ mμ than for $\lambda_1 = 375$ mμ.

Fig. 1 shows Stern-Volmer plots of representative series for both dyes and for the two pH values; the ionic strength, μ, is 0.045. Higher quencher concentrations /Q/, possible only in combination with higher μ, led to an upward curvature of the lines. It is seen that, for riboflavin, the quenching constant has the same value, $k^o = 30$ M^{-1}, at both pH values. For riboflavin phosphate, the quenching constant is higher at pH 5 ($k' = 23$ M^{-1}) than at pH 8 ($k'' = 0.79 . k'$). k^o and k' were found to be independent of μ, in contrast to k'' which is lower at lower μ (Fig. 2): for $\mu = 0.025$, $k'' = 0.70 . k'$. The μ value chosen for a series sets an upper limit to /Q/, i.e. to ($I_o/I - 1$). The experimental

errors therefore set a practical lower limit for μ.

Discussion

The light absorption /10/ and the fluorescence intensity /9/ of riboflavin is practically constant at least between pH 4 and pH 8, the same is true for riboflavin phosphate /2/. The two dyes are very similar both regarding light absorption and light emission properties. It therefore seems that the phosphate group, being separated from the iso-alloxazine group by the five-carbon aliphatic chain, has hardly any influence on the interaction of the dye with light. The ionization of the phosphate group does not affect the light absorption and would not be expected to affect light emission properties, such as the fluorescence lifetime or the sensitivity to the presence of a certain quencher at a certain position. - A study of the fluorescence lifetimes of the two dyes under different conditions (using a 15 Mc/s light source and a phase shift detector) is in progress /11/. A preliminary value for riboflavin phosphate at pH 7, $\tau_0 = 5.2$ nsec, coincides with a previous determination by Weber (cited by Gibson et al. /5/).

The quenching of riboflavin fluorescence by iodide was studied by Weber /13/. Our $\underline{k}^o$, reported above, is in fair agreement with the value that can be calculated from his data. - A plausible explanation for the difference between $\underline{k}^o$ and $\underline{k}'$ (which cannot be due to Coulomb forces) is the following: The phosphate group increases the size of the molecule, decreasing the diffusion constant $\underline{D}$ and thus the encounter rate. It also decreases the solid angle within which the quencher may approach the sensitive part of the molecule, which is equivalent to a decrease in γ. Both effects would be further accentuated if the phosphate were sur-

rounded by a fairly tightly bound shell of water molecules. The problem will not be pursued further in this paper.

The size of the riboflavin phosphate molecule should depend very little on whether or not the phosphate group is ionized. The reasoning from the preceding paragraph does not apply to the difference between $\underline{k}'$ and $\underline{k}''$, so the explanation in this case is most probably electrostatic effects. These will be of two types, "long range" and "local" effects. When the dye and the quencher are far from each other, they may be approximated as spheres with the charge (if any) located as the centre. The situation can be treated with standard theories for the diffusional movement of ions. Looking closer, the dye molecule may roughly be resembled to a disc (the iso-alloxazine group) with a radially extending rod (the ribityl group with the phosphate group at the upper end). When the phosphate group is charged, the probability of finding a negatively charged quencher near the far end of the rod is small, and the disc is partly protected from quencher approach towards the edge near the rod. The result can be expressed as a decrease in γ.

It seems likely that the "long range" and the "local" effects cooperate in making $\underline{k}''$ lower than $\underline{k}'$, but that primarily the "long range" effect is responsible for the diminuation of this decrease at higher ionic strengths.

Below, an attempt is made to compare, quantitatively, the different quenching constants with predictions based on a theory outlined by Förster /4/. It is likely that equally satisfactory results would be obtained using other theories, provided the various parameters were chosen properly.

The quenching constant is expected to follow the following equation:

$$\underline{k} = \gamma \cdot \frac{4\pi N_o}{1000} \cdot a \,(A.D.\, \tau_o + B.A \sqrt{D.\tau_o}\,) \tag{2}$$

where the quantities are to be expressed in c.g.s. units. $\underline{N}_o$ is Avogadro's number; the two parameters $\underline{A}$ and $\underline{B}$ are introduced to account for the electrostatic interaction and can be calculated from given equations (see below).

The diffusion constants for riboflavin and riboflavin phosphate are not known, but should be of the same magnitude as for fluorescein, or $0.4 \cdot 10^{-5}$ cm^2 sec^{-1} /7/. The diffusion constant for iodide is $2.0 \cdot 10^{-5}$ cm^2 sec^{-1} /6/, which would make $\underline{D} = 2.4 \cdot 10^{-5}$ cm^2 sec^{-1}. Together with $\tau_o = 5.2$ nsec, this gives $\underline{D}.\tau_o = 12.5 \cdot 10^{-14}$ cm and $\sqrt{D.\tau_o} = 35$ Å. - The corresponding Stokes' radii are 6.6 Å and 1.2 Å. The collision radius of the iodide ion should probably be increased to about 2.2 Å, which is the value obtained by X-ray diffraction for iodide ions in crystals /12/. The quenching distance $\underline{a}$ is probably of the same order as the sum of the two radii, or 8 to 10 Å.

For a neutral dye ($\underline{z}_A \cdot \underline{z}_Q = 0$), both $\underline{A}$ and $\underline{B}$ are equal to 1. For riboflavin ($\underline{k}^o = 30$ M^{-1}), Eq. (2) gives values of γ between 0.32 (for $\underline{a} = 8$ Å) and 0.25 (for $\underline{a} = 10$ Å). For unionized riboflavin phosphate ($\underline{k}' = 23$ M^{-1}), the corresponding γ values are 0.25 and 0.19.

The equations for calculating $\underline{A}$ and $\underline{B}$ contain, in addition to $\underline{z}_A$, $\underline{z}_Q$, and $\underline{a}$, also the temperature $\underline{T}$, the dielectric constant ϵ, and the ionic strength μ of the medium. For $\underline{z}_A \cdot \underline{z}_Q < 0$ (attraction), $\underline{B} > \underline{A} > 1$; for $\underline{z}_A \cdot \underline{z}_Q >$ (repulsion), $\underline{B} < \underline{A} < 1$. For aqueous solutions at room temperature, for $\underline{z}_A \cdot \underline{z}_Q = +1$,

and for $\underline{a}$ = 10 Å, Förster calculated $\underline{A}$ = 0.69 and $\underline{B}$ = 0.50 (for μ = 0) and $\underline{A}$ = 0.87 and $\underline{B}$ = 0.60 (for μ = 0.01). Additional values, calculated with the equations used by Förster, are collected in Fig. 3.

Defining $\underline{f}$ as the ratio between $\underline{k}''$ and $\underline{k}'$, Eq. (2) gives

$$f = \frac{k''}{k'} = \frac{\gamma'' \cdot a'' (A \cdot D'' \cdot \tau_0 + B \cdot a'' \sqrt{D'' \cdot \tau_0''})}{\gamma' \cdot a'(D'\tau_0' + a' \sqrt{D'\tau_0'})} \tag{3}$$

Assuming $\underline{a}'' = \underline{a}'$, $\underline{D}'' = \underline{D}'$, and $\tau_0'' = \tau_0'$, and introducing $\underline{g} = \gamma''/\gamma'$

$$\frac{g \cdot A - f}{f - g \cdot B} = \frac{a}{\sqrt{D\tau_0}} \tag{4}$$

Here, $\underline{A}$ and $\underline{B}$ account for the long range effect and $\underline{g}$ for the local effect of the electrostatic repulsion. Increasing μ leads to an increase in $\underline{f}$ (Fig. 2) and in $\underline{A}$ and $\underline{B}$ (Fig. 3). $\underline{D}$ and τ_0 are independent of μ, and the same is probably true for $\underline{g}$ and for $\underline{a}$. With the previously given values for $\underline{f}$, $\underline{D}$ and τ_0, and with $\underline{A}$ and $\underline{B}$ values taken from Fig. 3, Eq. (4) gives values for $\underline{a}$ for any chosen value of $\underline{g}$. A few examples are given in Table I. The agreement between $\underline{a}$ values for the two series (with different μ) is surprisingly good. It is seen that $\underline{a}$ = 8 to 10 Å corresponds to a $\underline{g}$ value around 0.8.

Even apart from the uncertainty in $\underline{D}$ and τ_0, there are reasons to judge the quantitative results above with caution. $\underline{A}$ values above 1, as found in Fig. 3, are in fact physically impossible (the implication would be that the electrostatic repulsion leads to an increased encounter rate). The derivation of the equations used in calculating $\underline{A}$ and $\underline{B}$ contain some approximations, which evidently are not valid at the comparatively

high ionic strengths used in the present experiments. Further work should therefore be extended to lower ionic strength (possible by averaging out experimental errors among a larger amount of data). Measurements should also be done, varying the temperature as well as the dielectric constant of the medium (using mixtures of water and, e.g., ethanol or dioxane).

Table I

		$\mu = 0.025$	$\mu = 0.045$
		$\underline{f} = 0.70$	$\underline{f} = 0.79$
$\underline{g} = 1.00$		$\underline{a} = 6.10 \pm 0.05$	$\underline{a} = 6.10 \pm 0.05$
0.90		7.25	7.25
0.80		9.20	9.15
0.75		11.4 ± 0.1	11.6 ± 0.1

The influence on the reaction rate from electrostatic forces has also been measured for other reactions with low activation energy (cf. a recent review /3/). Again, there is the difficulty of separating the encounter frequency from the efficiency of each encounter. For addition of a proton ($\underline{z}_1 = +1$) to a series of molecules with similar steric configuration and charge $\underline{z}_2$, the rates were found to decrease as 7.5, 2.6, 1 , 0.48 ($\times 10^{10}$ M^{-1} sec^{-1}) when $\underline{z}_2$ increases as -1, 0, +1, +2. The disproportionation of duro semiquinone anion is appreciably slower ($4.6 \cdot 10^6$ M^{-1} sec^{-1}) than that of the neutral duro semiquinone ($8. \cdot 10^8$ M^{-1} sec^{-1}) /1/. A similar difference, although less pronounced, is found for the disproportionation of the semiquinone of riboflavin phosphate: $1 \cdot 10^8$ M^{-1} sec^{-1} for the anion compared with $4 \cdot 10^8$ M^{-1} sec^{-1} for the neutral form /8/. The situation

in these cases is not easily interpreted, as the disproportionation
for the semiquinone anion involves an electron transfer, but for
the neutral semiquinone a hydrogen transfer.

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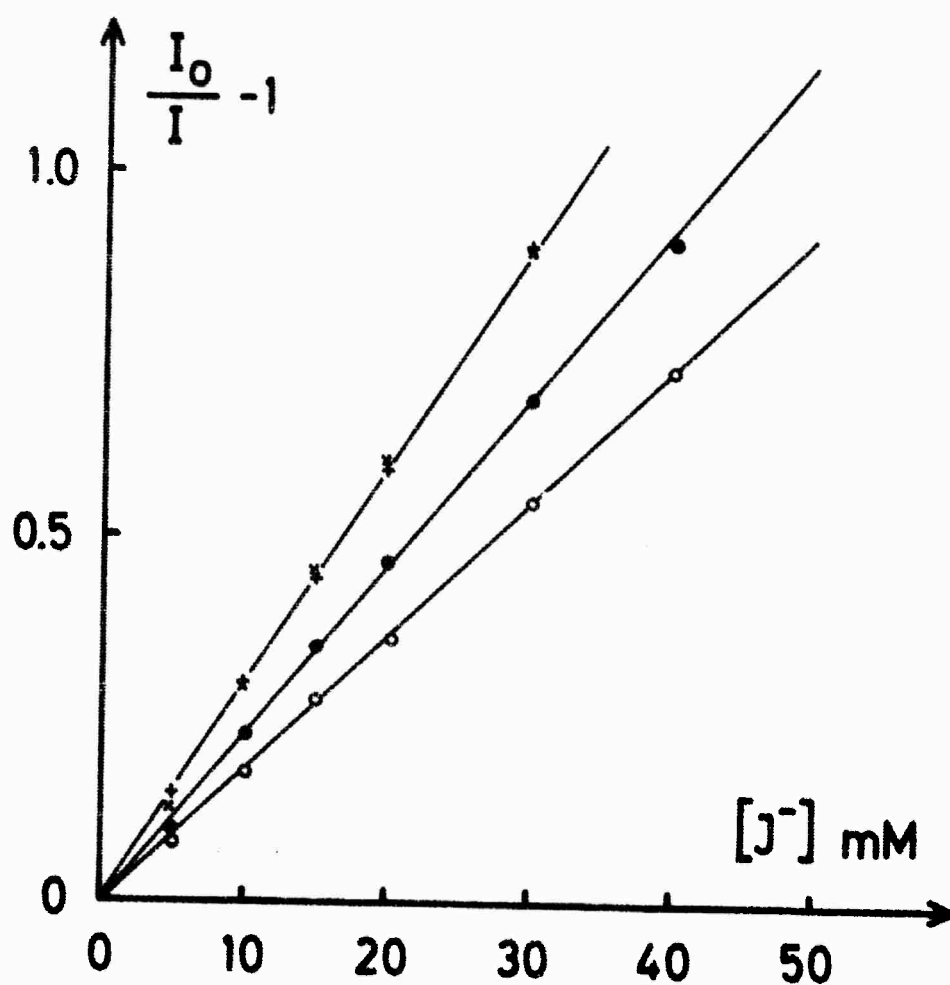
Text to the figures

Fig. 1. Stern-Volmer plot of the iodide quenching of the
fluorescence of riboflavin (at pH 5.1 x, and at pH
7.9 +) and of riboflavin phosphate (at pH 5.1 ● and
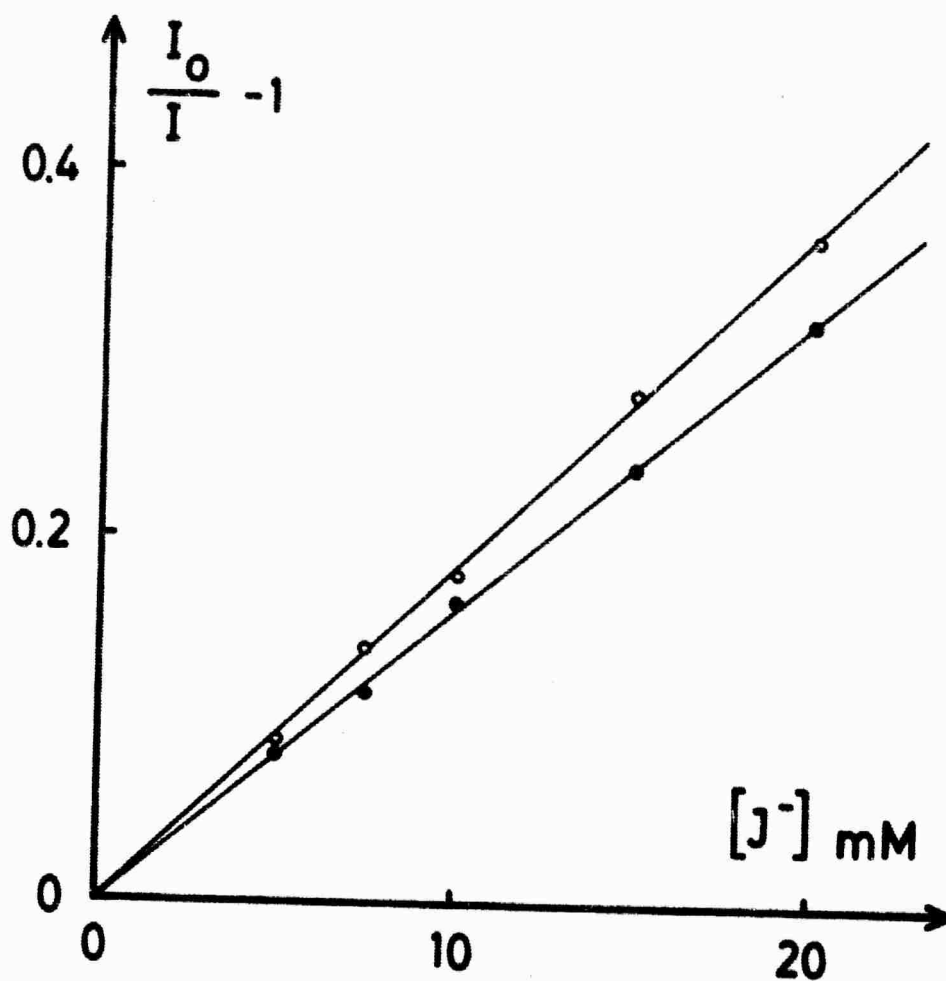at pH 7.9 o). Ionic strength 0.045.

Fig. 2. Stern-Volmer plot of iodide quenching of the
fluorescence of riboflavin phosphate at pH 7.9.

● $\mu = 0.025$ o $\mu = 0.045$.

Fig. 3. The parameters A and B, calculated from equations
given by Förster /4/, for different ionic strengths μ
and different quenching distances $\underline{a}$.

Fig. 1 Stern-Volmer plot of the iodide quenching of the
fluorescence of riboflavin (at pH 5.1 x, and at
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Fig. 2 Stern-Volmer plot of iodide quenching of the
fluorescence of riboflavin phosphate at
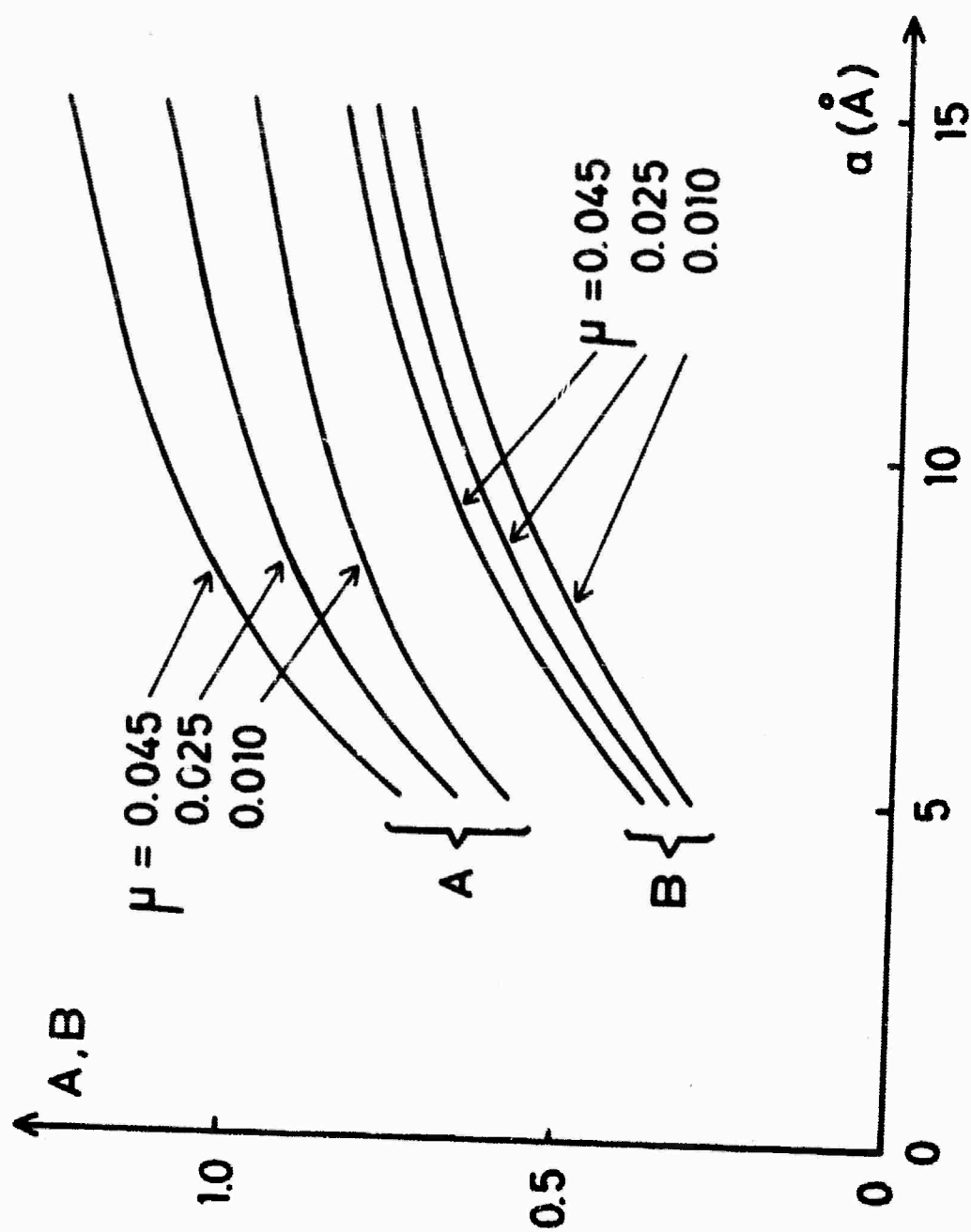pH 7.9. ● μ= 0.025, o μ= 0.045.

Fig. 3 The parameters A and B, calculated from equations
given by Förster /4/, for different ionic strengths
μ and different quenching distances a.